Tenacibaculum aiptasiae sp. nov., isolated from a sea anemone *Aiptasia pulchella*

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A novel bacterial strain, designated a4^T, isolated from a sea anemone (*Aiptasia pulchella*) in Taiwan, was characterized using a polyphasic taxonomic approach. Strain a4^T was aerobic, Gram-negative, pale-yellow-pigmented and rod-shaped. It grew optimally at 30–35 °C, in the presence of 3–4 % (w/v) NaCl and at pH 8.0. Phylogenetic analyses based on 16S rRNA gene sequences showed that the strain belonged to the genus *Tenacibaculum* (family *Flavobacteriaceae*, phylum *Bacteroidetes*). The closest neighbours were *Tenacibaculum lutimaris* TF-26^T (97.6 % similarity) and *Tenacibaculum aestuarii* SMK-4^T (97.7 % similarity). The novel isolate could be distinguished from all *Tenacibaculum* species by several phenotypic characteristics. The major fatty acids were summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, 19.6 %), iso-$C_{15:0}$ (12.9 %), iso-$C_{16:0}$ 3-OH (10.2 %), iso-$C_{17:0}$ 3-OH (9.9 %) and iso-$C_{15:1}$ (9.5 %). The DNA G+C content was 35.0 mol%. Hence, genotypic and phenotypic data demonstrate that strain a4^T should be classified as a representative of a novel species in the genus *Tenacibaculum*, for which the name *Tenacibaculum aiptasiae* sp. nov. is proposed. The type strain is a4^T (=BCRC 17655^T =LMG 24004^T).

The genus *Tenacibaculum* (Suzuki *et al.*, 2001) is a member of the family *Flavobacteriaceae* (Reichenbach, 1992a, b; Bernardet *et al.*, 1996, 2002; Bernardet & Nakagawa, 2006) and encompasses Gram-negative, non-spore-forming, yellow-pigmented, straight rods with a DNA G+C content of 30.0–35.2 mol% (Suzuki *et al.*, 2001; Frette *et al.*, 2004; Yoon *et al.*, 2005; Choi *et al.*, 2006; Sheu *et al.*, 2007). It currently contains nine species isolated from different marine sources, including *Tenacibaculum maritimum*, *T. ovolyticum*, *T. mesophilum*, *T. amylolyticum*, *T. skagerrakense*, *T. lutimaris*, *T. litoreum*, *T. aestuarii* and *T. litopenaei* (Wakabayashi *et al.*, 1986; Hansen *et al.*, 1992; Suzuki *et al.*, 2001; Frette *et al.*, 2004; Yoon *et al.*, 2005; Choi *et al.*, 2006; Jung *et al.*, 2006; Sheu *et al.*, 2007).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Tenacibaculum aiptasiae* a4^T is EF416572.

A comparison of the fatty acid composition of strain a4^T and related type strains, the MicroPlate GN2 profile of strain a4^T and a 16S rRNA gene sequence-based phylogenetic tree including two novel *Tenacibaculum* species, details of which were published after submission of this paper, are available as supplementary material with the online version of this paper.

Aiptasia pulchella is a sea anemone that is widely distributed in the tropical and subtropical Pacific Ocean. It is easily cultured in the laboratory and commonly used as a model animal for studies of the *Symbiodinium*–cnidarian symbiosis (Wang & Douglas, 1997, 1998, 1999). However, it occasionally becomes unhealthy for unknown reasons when maintained in a closed system. In this study, the taxonomic position of a novel organism, designated strain a4^T, which was isolated from a diseased sea anemone, is described.

In order to isolate micro-organisms from a sea anemone maintained in an aquarium in Pingtung, Taiwan, the animal was surface-sterilized with 75 % ethanol for 30 s, rinsed six times in sterile artificial seawater (NaCl, 24 g; $MgCl_2$, 5.1 g; Na_2SO_4, 4 g; $CaCl_2$, 1.1 g; KCl, 0.7 g; $NaHCO_3$, 0.2 g; KBr, 0.1 g; H_3BO_3, 0.027 g; $SrCl_2$, 0.024 g; NaF, 0.003 g; distilled water, 1 l; Lyman & Fleming, 1940), crushed and streaked on marine 2216 agar (MA; BD Difco) plates incubated at 25 °C. After primary isolation, strain a4^T was subcultivated on MA or in marine 2216 broth (MB; BD Difco). The strain was preserved as a suspension in 20 % (v/v) glycerol MB or by lyophilization

in 20 % (w/v) skimmed milk MB. The organism was subjected to a polyphasic taxonomic study.

The morphology of bacterial cells was observed in an MB culture grown at 25 °C for 6 h (lag growth phase), 18 h (exponential phase) and 36 h (stationary phase) under a phase-contrast microscope (Leica DM 2000). Gliding motility was tested as described by Bowman (2000) and Bernardet *et al.* (2002) using an MB culture grown for 24 and 48 h at 25 °C. Flagellar staining was performed using the Spot Test flagella stain (BD Difco). The Gram reaction was performed using the Gram stain set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995). Accumulation of poly-β-hydroxybutyrate granules was observed by light microscopy after staining cells with Sudan black. Colony morphology was examined using a stereoscopic microscope (Nikon SMZ 800). Cellular pigments were extracted with acetone/methanol (7 : 2, v/v), from cultures grown on MA and absorption spectra were determined with a scanning UV/visible spectrophotometer (Helios Delta; Thermo Fisher Scientific). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992a) and Bernardet *et al.* (2002). The optimum pH range for growth was examined in MB using appropriate biological buffers such as glycine/HCl, citrate/Na_2HPO_4, phosphate buffer and glycine/NaOH for adjusting the pH to 3.0–4.0, 4.0–8.0, 6.0–8.0 and 9.0–11.0 (at 1.0 pH unit intervals), respectively. The pH values were adjusted prior to sterilization and post-sterilization controls revealed that only minor changes in pH had occurred. The NaCl requirement was determined using nutrient broth (BD Difco) containing 0, 0.5 and 1.0–10.0 % (w/v) NaCl (at 1.0 % intervals). The temperature range for growth was examined at 4, 8, 10, 15, 20, 25, 30, 35, 40 and 42 °C in MB adjusted to pH 7.0 using an orbital water-bath shaker (125 r.p.m.). Growth rate was determined by measuring the turbidity (OD_{600}) of cultures grown at various pH, NaCl concentrations and temperatures. Anaerobic growth was tested on MA using the Oxoid AnaeroGen system. Catalase, oxidase and DNase activities and hydrolysis of starch, casein, chitin, aesculin, gelatin and Tweens 20, 40, 60 and 80 were determined using standard methods (Gerhardt *et al.*, 1994; MacFaddin, 2000; Chang *et al.*, 2004). The commercially available API 20NE (bioMérieux), API ZYM (bioMérieux) and MicroPlate GN2 (Biolog) microtest systems were used, according to the manufacturers' instructions, to determine the biochemical properties, enzyme activities and carbohydrate utilization pattern of strain a4^T. The API ZYM strip was read after 4 h incubation at 37 °C, whereas API 20NE and MicroPlate GN2 were read after 72 h at 25 °C. The three commercial systems were inoculated with a cell suspension in artificial seawater. Sensitivity of strain a4^T to different antibiotics was analysed by the diffusion method on MA. The following antibiotic discs (Oxoid) were used: ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), rifampicin (5 μg), penicillin G (10 U), streptomycin (10 μg) and tetracycline (30 μg). The effect of antibiotics on cell growth was assessed after 2 days incubation at 25 °C and susceptibility was scored based on the distance from the edge of the clear zone to the disc.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chen *et al.* (2001). Sequences were assembled by using the Fragment Assembly System program of the Wisconsin Package 8.1 (GCG, 1995). Multiple-sequence alignments of strain a4^T and its closest relatives were performed using BIOEDIT (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred using the maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. An evolutionary-distance matrix was generated for the neighbour-joining algorithm using the Jukes & Cantor (1969) distance model and bootstrap analysis (1000 resamplings).

The nearly complete 16S rRNA gene sequence (1434 nt) of strain a4^T was obtained. Its comparison with the sequences of representative members of genera in the family *Flavobacteriaceae* showed that this organism fell within the evolutionary radiation of the genus *Tenacibaculum* (Fig. 1). Sequence similarity calculations using the pairwise alignment obtained from the EzTaxon database (Chun *et al.*, 2007) showed that strain a4^T shared the greatest degree of 16S rRNA gene sequence similarity with *T.*

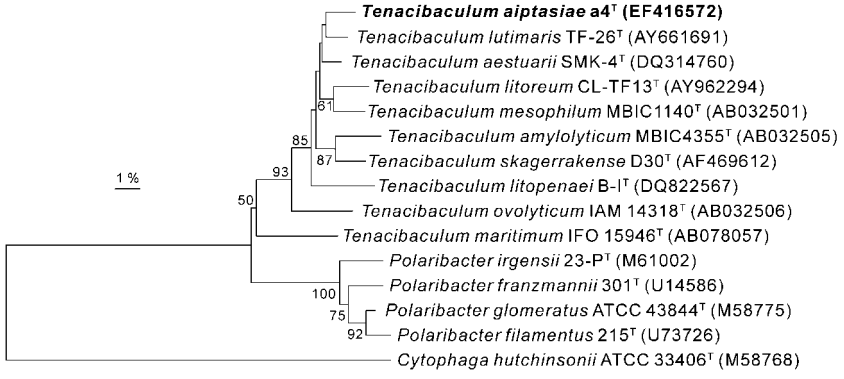

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain a4^T, other *Tenacibaculum* species and related taxa. Sequences were retrieved from EMBL (accession numbers are given in parentheses). Numbers at nodes are bootstrap values (>50 %) based on 1000 resampled datasets. *Cytophaga hutchinsonii* ATCC 33406^T was used as an outgroup. The maximum-parsimony tree showed a very similar topology (not shown). Bar, 1 % sequence dissimilarity.

Table 1. Phenotypic characteristics of strain a4^T and other *Tenacibaculum* species

Strains: 1, *T. aiptasiae* sp. nov. a4^T; 2, *T. litopenaei* B-I^T; 3, *T. amylolyticum* MBIC 4355^T; 4, *T. aestuarii* SMK-4^T; 5, *T. litoreum* CL-TF13^T; 6, *T. lutimaris* TF-26^T; 7, *T. maritimum* NCIMB 2154^T; 8, *T. mesophilum* MBIC1140^T; 9, *T. ovolyticum* IAM 14318^T; 10, *T. skagerrakense* D30^T. Data for reference strains were taken from Sheu *et al.* (2007), Jung *et al.* (2006), Choi *et al.* (2006), Yoon *et al.* (2005), Frette *et al.* (2004) and Suzuki *et al.* (2001). Colony and cell morphology of the strains were not all determined on the same media; consult original data for direct comparisons. +, Positive; −, negative; W, weakly positive; NA, not available; NG, no growth with NaCl only.

Characteristic	1	2	3	4	5	6	7	8	9	10
Origin	Sea anemones, Taiwan	Shrimp culture pond, Taiwan	Macroalgae, Japan	Tidal flat, Korea	Tidal flat, Korea	Tidal flat, Korea	Diseased Red sea bream, Japan	Sponge and macroalgae, Japan	Halibut egg, Norway	Pelagic, Denmark
Colony morphology										
Shape	Circular, convex, spreading edge	Circular, convex, spreading edge	Circular, spreading edge	Irregular, spreading edge	Irregular, spreading edge	Irregular, spreading edge	Uneven edge	Irregular, spreading edge	Regular edge	Circular, convex, spreading edge
Diameter at 5 days (mm)	10–20	5–20	23–27	5–10	5–10	10–20	<5	30–60	NA	5–20
Colour	Pale yellow	Yellow	Yellow	Pale yellow	Pale yellow	Pale yellow	Pale yellow	Yellow	Pale yellow	Bright yellow
Cell size (μm)	0.4–0.6 × 2–7	0.3–0.5 × 2–10	0.4 × 2–5	0.3 × 2–3.5	0.3–0.5 × 2–35	0.5 × 2–10	0.5 × 2–30	0.5 × 1.5–10	0.5 × 2–20	0.5 × 2–15
Spherical cells in ageing broth cultures	Very rare	Very rare	Very rare	NA	Very rare	Very rare	Frequent	Very rare	Absent	Frequent
Gliding motility	+	+	+	+	+	+	+	+	+	−
Salinity tolerance (NaCl; %)	1–10	2–10	3 (W)	<7	3–5	<8	NG	1–7	NG	NG
Growth at pH 5	−	+	−	−	−	+	−	−	−	−
Nitrate reduction	−	−	W	−	+	−	+	−	+	+
Hydrolysis of:										
Chitin	−	+	−	NA	NA	NA	−	−	+	−
Starch	+	+	+	−	+	−	−	−	−	+
Gelatin	+	+	+	+	+	+	−	+	+	NA
Tween 80	+	−	+	+	+	−	+	+	+	−
Carbon source utilization										
Citrate	−	−	−	NA	−	NA	−	−	−	+
L-Leucine	−	−	−	−	−	−	−	−	−	W
L-Proline	−	+	+	−	+	−	−	+	−	+
L-Glutamate	+	+	+	−	−	−	W	+	−	+
L-Aspartate	+	−	−	−	−	−	−	+	−	+
D-Glucose	−	+	NA	−	−	−	NA	NA	NA	+
Sucrose	−	−	−	−	−	−	−	−	−	+
DNA G+C content (mol%)	35.0	35.2	30.9	33.6	30	32.6	31.6	31.8	30.3	35.2

lutimaris TF-26^T (97.6 %) and *T. aestuarii* SMK-4^T (97.7 %). Strain a4^T shared lower 16S rRNA gene sequence similarities with the type strains of *T. litoreum* (96.9 %), *T. mesophilum* (96.7 %), *T. skagerrakense* (96.6 %), *T. ovolyticum* (96.4 %), *T. amylolyticum* (95.6 %), *T. litopenaei* (95.5 %) and *T. maritimum* (94.8 %). Similarity levels of strain a4^T with other bacterial species in the family *Flavobacteriaceae* were less than 94.5 %.

DNA–DNA hybridization experiments were performed with photobiotin-labelled probes using the fluorometric method of Ezaki *et al.* (1989). Hybridizations were conducted in 50 % formamide at 50 °C. Reciprocal reactions were also performed and all experiments were duplicated. Strain a4^T shared only low levels of DNA relatedness with its closest phylogenetic neighbours *T. lutimaris* TF-26^T (28 ± 5 %; reciprocal, 21 ± 6 %) and *T. aestuarii* SMK-4^T (23 ± 7 %; reciprocal, 19 ± 2 %), clearly indicating that it represents a novel species in the genus *Tenacibaculum*.

Fatty acids were extracted from cells grown on MA for 48 h at 28 °C and prepared by the standard protocol of the Microbial Identification System (MIDI; Microbial ID) (Sasser, 1990). The fatty acid composition of strain a4^T was dominated by summed feature 3 (comprising $C_{16:1}$ $\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 19.6 %), iso-$C_{15:0}$ (12.9 %), iso-$C_{16:0}$ 3-OH (10.2 %), iso-$C_{17:0}$ 3-OH (9.9 %) and iso-$C_{15:1}$ (9.5 %). The detailed fatty acid composition of strain a4^T, available in Supplementary Table S1 in IJSEM Online, was in accordance with those of other *Tenacibaculum* species (Jung *et al.*, 2006; Choi *et al.*, 2006; Yoon *et al.*, 2005; Sheu *et al.*, 2007). Menaquinones were extracted and analysed by HPLC as described by Collins *et al.* (2000). The predominant quinone of strain a4^T was a menaquinone with six isoprenoid units (MK-6). The DNA G+C content of strain a4^T, determined in triplicate as described by Mesbah *et al.* (1989), was 35.0 ± 1.0 mol%, which is within the range reported previously for *Tenacibaculum* species (30.0–35.2 mol%; Table 1).

Detailed results of the phenotypic study are provided in Table 1 and in the species description; MicroPlate GN2 data are available in Supplementary Table S2. Results indicated that strain a4^T could be distinguished from its phylogenetic relatives using a combination of phenotypic properties, especially nitrate reduction, hydrolysis of chitin, starch, gelatin and Tween 80 and utilization of various organic compounds.

Hence, genotypic and phenotypic data support the description of a novel species in the genus *Tenacibaculum* for strain a4^T. The name *Tenacibaculum aiptasiae* sp. nov. is proposed for this taxon.

After submission of this manuscript, Piñeiro-Vidal *et al.* (2008) described two novel species of the genus *Tenacibaculum*. The 16S rRNA gene sequence similarity values of the sequence of strain a4^T to the type strains of these two species are 96.8 % to *Tenacibaculum discolor* LL04 11.1.1^T and 96.6 % to *Tenacibaculum gallaicum* A37.1^T. To evaluate the position of all known *Tenacibaculum* species, a new phylogenetic dendrogram based on 16S rRNA gene sequences was constructed (Supplementary Fig. S1); in this tree, strain a4^T, *T. discolor* LL04 11.1.1^T and *T. gallaicum* A37.1^T form three distinct branches within the genus *Tenacibaculum*.

Description of *Tenacibaculum aiptasiae* sp. nov.

Tenacibaculum aiptasiae (aip.ta′si.ae. N.L. n. *Aiptasia* the scientific name of a genus of sea anemone; N.L. gen. n. *aiptasiae* isolated from a sea anemone belonging to the genus *Aiptasia*).

Cells are aerobic, Gram-negative, non-flagellated, non-spore-forming, straight rods (0.4–0.6 μm in width and 2.0–7.0 μm in length) that are motile by gliding. Degenerative spherical cells are occasionally observed in ageing cultures in broth. Poly-β-hydroxybutyrate granules are stored as reserve material. Colonies are pale yellow, circular and convex with spreading margins and approximately 10–20 mm in diameter after 5 days incubation at 25 °C on MA. Flexirubin-type pigments are absent (KOH test negative). Carotenoid pigments are present with main absorption peaks at 480, 454 and 425 nm. Growth occurs at 8–40 °C, in the presence of 1–10 % NaCl and at pH 7–9. Optimum growth occurs at 30–35 °C, with 3–4 % NaCl and at pH 8.0. Starch, DNA, casein, aesculin, gelatin and Tweens 40, 60 and 80 are hydrolysed, but chitin and Tween 20 are not. Oxidase and catalase activities are present. In the API 20NE strip, the reaction is positive for aesculin and gelatin hydrolysis, but negative for nitrate reduction, indole production, glucose fermentation, urease, arginine dihydrolase, β-galactosidase and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenylacetate. In the API ZYM strip, alkaline phosphatase, C4 esterase, C8 lipase, C14 lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present, but α-galactosidase, β-galactosidase, β-glucosidase, α-glucosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. Resistant to gentamicin, kanamycin and streptomycin; sensitive to ampicillin, chloramphenicol, nalidixic acid, novobiocin, penicillin G, rifampicin and tetracycline. The major fatty acids (>9 %) are summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), iso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH, iso-$C_{17:0}$ 3-OH and iso-$C_{15:1}$. The predominant quinone is MK-6.

The type strain is a4^T (=BCRC 17655^T=LMG 24004^T), isolated from a sea anemone *Aiptasia pulchella* cultured in a laboratory in Taiwan. The DNA G+C content of the type strain is 35.0 mol%.

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